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(54) Title: CHIMERIC FLAVIVIRUS VACCINES

(57) Abstract

A chimeric live, infectious, attenuated virus, containing a yellow fever virus, in which the nucleotide sequence for a prM-E protein is either deleted, truncated, or mutated, so that functional prM-E protein is not expressed, and integrated into the genome of the yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that the prM-E protein of the second flavivirus is expressed.

CHIMERIC FLAVIVIRUS VACCINES

Background of the Invention

5   This invention relates to infectious, attenuated viruses useful as

vaccines against diseases caused by flaviviruses.

Several members of the flavivirus family pose current or potential

threats to global public health. For example, Japanese encephalitis is a

significant public health problem involving millions of at risk individuals in the

10   Far East. Dengue virus, with an estimated annual incidence of 100 million

cases of primary dengue fever and over 450,000 cases of dengue hemorrhagic

fever worldwide, has emerged as the single most important arthropod-

transmitted human disease.   Other flaviviruses continue to cause endemic

diseases of variable nature and have the potential to emerge into new areas as a

15   result of changes in climate, vector populations, and environmental

disturbances caused by human activity. These flaviviruses include, for

example, St. Louis encephalitis virus, which causes sporadic, but serious acute

disease in the midwest, southeast, and western United States; West Nile virus,

which causes febrile illness, occasionally complicated by acute encephalitis,

20   and is widely distributed throughout Africa, the Middle East, the former Soviet

Union, and parts of Europe; Murray Valley encephalitis virus, which causes

endemic nervous system disease in Australia; and Tick-borne encephalitis

virus, which is distributed throughout the former Soviet Union and eastern

Europe, where its ixodid tick vector is prevalent and responsible for a serious

25   form of encephalitis in those regions.

Hepatitis C virus (HCV) is another member of the flavivirus family,

with a genome organization and replication strategy that are similar, but not

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virions by a furin-like cellular protease (Stadler *et al.*, J. Virol. 71:8475-8481, 1997), which is necessary to activate hemagglutinating activity, fusogenic activity, and infectivity of virions. The M protein is cleaved from its precursor protein (prM) after the consensus sequence R-X-R/K-R (X is variable), and

5   incorporated into the virus lipid envelope together with the E protein.

Cleavage sequences have been conserved not only within flaviviruses, but also within proteins of other, unrelated viruses, such as PE2 of murine coronaviruses, PE2 of alphaviruses, HA of influenza viruses, and p160 of retroviruses. Cleavage of the precursor protein is essential for virus

10   infectivity, but not particle formation. It was shown that, in case of a TBE-dengue 4 chimera, a change in the prM cleavage site resulted in decreased neurovirulence of this chimera (Pletnev *et al.*, J. Virol. 67:4956-4963, 1993), consistent with the previous observation that efficient processing of the prM is necessary for full infectivity (Guirakhoo *et al.*, 1991, *supra*, 1992, *supra*; Heinz

15   *et al.*, Virology 198:109-117, 1994). Antibodies to prM protein can mediate protective immunity, apparently due to neutralization of released virions that contain some uncleaved prM. The proteolytic cleavage site of the PE2 of VEE (4 amino acids) was deleted by site-directed mutagenesis of the infectious clone (Smith *et al.*, ASTMH meeting, December 7-11, 1997). Deletion mutants

20   replicated with high efficiency and PE2 proteins were incorporated into particles. This mutant was evaluated in non-human primates and shown to cause 100% seroconversion and protected all immunized monkeys from a lethal challenge.

Summary of the Invention

25   The invention features chimeric, live, infectious, attenuated viruses that are each composed of:

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Japanese Encephalitis (JE), Dengue (DEN, *e.g.*, any of Dengue types 1-4), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE), and Hepatitis C (HCV) viruses. Additional flaviviruses for use as the second flavivirus include Kunjin virus,

5   Central European Encephalitis virus, Russian Spring-Summer Encephalitis virus, Powassan virus, Kyasanur Forest Disease virus, and Omsk Hemorrhagic Fever virus. In a preferred chimeric virus of the invention, the prM-E protein coding sequence of the second flavivirus is substituted into the prM-E protein coding sequence of the live yellow fever virus. In a preferred chimeric virus,

10   the prM-E protein coding sequence is derived from an attenuated virus strain, such as a vaccine strain. Also, as is described further below, the prM portion of the protein can contain a mutation that prevents cleavage to generate mature membrane protein.

        Also included in the invention are methods of preventing or treating

15   flavivirus infection in a mammal, such as a human, by administering a chimeric flavivirus of the invention to the mammal; use of the chimeric flaviviruses of the invention in the preparation of medicaments for preventing or treating flavivirus infection; nucleic acid molecules encoding the chimeric flaviviruses of the invention; and methods of manufacturing the chimeric flaviviruses of the

20   invention.

        The invention provides several advantages. For example, because they are live and replicating, the chimeric viruses of the invention can be used to produce long-lasting protective immunity. Because the viruses have the replication genes of an attenuated virus (*e.g.*, Yellow Fever 17D), the resulting

25   chimeric virus is attenuated to a degree that renders it safe for use in humans.

        Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

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Detailed Description

The invention provides chimeric flaviviruses that can be used in

vaccination methods against flavivirus infection. Construction and analysis of

chimeric flaviviruses of the invention, such as chimeras of yellow fever virus

5   and Japanese Encephalitis (JE), Dengue types 1-4 (DEN 1-4), Murray Valley

Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-

borne Encephalitis (TBE), and Hepatitis C (HCV) viruses are described as

follows.

Flavivirus proteins are produced by translation of a single, long open

10   reading frame (encoding, *i.a.*, the structural proteins, capsid (C), pre-membrane

(pr-M), and envelope (E), as well as non-structural proteins) and a complex

series of post-translational proteolytic cleavages. The chimeric flaviviruses of

the invention, as is discussed above, include those in which the pr-M and E

proteins of one flavivirus have been replaced by the pr-M and E proteins of

15   another flavivirus. Thus, creation of these chimeric flaviviruses involves the

generation of novel junctions between the capsid and pre-membrane proteins,

and the envelope protein and the non-structural region (NS1), of two different

flaviviruses. Cleavage between each of these sets of proteins (C and pr-M, and

E and NS1) occurs during the natural proteolytic processing of flavivirus

20   proteins, and requires the presence of signal sequences flanking the junctions of

the cleavage sites.

In the chimeric flaviviruses of the invention, it is preferred that the

signal sequences of the viruses making up the chimeras are substantially

maintained, so that proper cleavage between the C and pr-M and E and NS1

25   proteins can efficiently take place. These signal sequences have been

maintained in the chimeras described below. Alternatively, any of numerous

known signal sequences can be engineered to link the C and pre-M or E and

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JE sequences from the start of the prM protein (nucleotide 478, amino acid

128) through the E/NS1 cleavage site (nucleotide 2,452, amino acid 817). In

addition to cloning of JE cDNA, several steps were required to introduce or

eliminate restriction sites in both the YF and JE sequences to permit *in vitro*

5   ligation. The structure of the template for regenerating chimeric YF (C)/JE

(prM-E) virus is shown in Fig. 4. A second chimera, encoding the entire JE

structural region (C-prM-E) was engineered using a similar strategy.

Molecular Cloning of the JE Virus Structural Region

          Clones of authentic JE structural protein genes were generated from

10   the JE SA_{14}-14-2 strain (JE live, attenuated vaccine strain), because the

biological properties and molecular characterization of this strain are well-

documented (see, *e.g.*, Eckels *et al.*, Vaccine 6:513-518, 1988; JE SA_{14}-14-2

virus is available from the Centers for Disease Control, Fort Collins, Colorado

and the Yale Arbovirus Research Unit, Yale University, New Haven,

15   Connecticut, which are World Health Organization-designated Reference

Centers for Arboviruses in the United States). JE SA_{14}-14-2 virus at passage

level PDK-5 was obtained and passaged in LLC-MK_2 cells to obtain sufficient

amounts of virus for cDNA cloning. The strategy we used involved cloning the

structural region in two pieces that overlap at an *Nhe*I site (JE nucleotide

20   1,125), which can then be used for *in vitro* ligation.

          RNA was extracted from monolayers of infected LLC-MK_2 cells and

first strand synthesis of negative sense cDNA was carried out using reverse

transcriptase with a negative sense primer (JE nucleotide sequence 2,456-71)

containing nested *Xba*I and *Nar*I restriction sites for cloning initially into

25   pBluescript II KS(+), and subsequently into YFM5.2(*Nar*I), respectively. First

strand cDNA synthesis was followed by PCR amplification of the JE sequence

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positive sense primer corresponding to YF5'3'IV nucleotides 6,625-6,639 to

generate PCR fragments that were then used as negative sense PCR primers in

conjunction with a positive sense primer complementary to the pBluescript

vector sequence upstream of the *EcoR*I site, to amplify the JE sequence

5   (encoded in reverse orientation in the pBluescript vector) from nucleotide 477

(N-terminus of the prM protein) through the *Nhe*I site at nucleotide 1,125. The

resulting PCR fragments were inserted into the YF5'3'IV plasmid using the

*Not*I and *EcoR*I restriction sites. This construct contains the SP6 promoter

preceding the YF 5'-untranslated region, followed by the sequence: YF (C) JE

10   (prM-E), and contains the *Nhe*I site (JE nucleotide 1,125) required for *in vitro*

ligation.

Engineering YFM5.2 and YF5'3'IV to Contain Restriction Sites for in vitro

Ligation

In order to use the *Nhe*I site within the JE envelope sequence as a 5'

15   *in vitro* ligation site, a redundant *Nhe*I site in the YFM5.2 plasmid (nucleotide

5,459) was eliminated. This was accomplished by silent mutation of the YF

sequence at nucleotide 5,461 (T→C; alanine, amino acid 1820). This site was

incorporated into YFM5.2 by ligation of appropriate restriction fragments and

introduced into YFM5.2(*Nar*I)/JE by exchange of an *Nsi*I/*Nar*I fragment

20   encoding the chimeric YF/JE sequence.

To create a unique 3' restriction site for *in vitro* ligation, a *BspE*I site

was engineered downstream of the *Aat*II site normally used to generate full-

length templates from YF5'3'IV and YFM5.2. (Multiple *Aat*II sites are present

in the JE structural sequence, precluding use of this site for *in vitro* ligation.)

25   The *BspE*I site was created by silent mutation of YF nucleotide 8,581 (A→C;

serine, amino acid 2,860) and was introduced into YFM5.2 by exchange of

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YFM5.2/JE are digested with *NheI/BspEI* and *in vitro* ligation is performed

using 50 nanograms of purified fragments in the presence of T4 DNA ligase.

The ligation products are linearized with *XhoI* to allow run-off transcription.

SP6 transcripts are synthesized using 50 nanograms of purified template,

5   quantitated by incorporation of ^{3}H-UTP, and integrity of the RNA is verified by

non-denaturing agarose gel electrophoresis. Yields range from 5 to 10

micrograms of RNA per reaction using this procedure, most of which is present

as full-length transcripts. Transfection of RNA transcripts in the presence of

cationic liposomes is carried out as described by Rice *et al.* (*supra*) for YF17D.

10   In initial experiments, LLC-MK$_2$ cells were used for transfection and

quantitation of virus, since we have determined the permissiveness for

replication and plaque formation of the parental strains of YF and JE. Table 1

illustrates typical results of transfection experiments using Lipofectin

(GIBCO/BRL) as a transfection vehicle. Vero cell lines have also been used

15   routinely for preparation of infectious virus stocks, characterization of labeled

proteins, and neutralization tests.

Nucleotide Sequencing of Chimeric cDNA Templates

       Plasmids containing the chimeric YF/JE cDNA were subjected to

sequence analysis of the JE portion of the clones to identify the correct

20   sequences of the SA$_{14}$-14-2 and Nakayama envelope protein. The nucleotide

sequence differences between these constructs in comparison to the reported

sequences (McAda *et al.*, *supra*) are shown in Table 2.

Structural and Biological Characterization of Chimeric YF/JE Viruses

       The genomic structure of chimeric YF/JE viruses recovered from

25   transfection experiments was verified by RT/PCR-based analysis of viral RNA

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monoclonal antibodies in this analysis. Plaque reduction neutralization testing
(PRNT) was performed on the chimeric viruses and the YF and JE SA$_{14}$-14-2
viruses using YF and JE-specific hyperimmune ascitic fluid (ATCC) and YF-
specific purified IgG (monoclonal antibody 2E10). Significant differences in

5   the 50% plaque reduction titer of these antisera were observed for the chimeras
when compared to the control viruses in these experiments (Table 3). Thus,
epitopes required for neutralization are expressed in the infectious chimeric
YF/JE viruses.

- -

Growth Properties in Cell Culture

10   The growth capacity of the chimeras has been examined
quantitatively in cell lines of both primate and mosquito origin. Fig. 2
illustrates the cumulative growth curves of the chimeras on LLC-MK$_2$ cells
after low multiplicity infection (0.5 plaque-forming units/cell). In this
experiment, YF5.2iv (cloned derivative) and JE SA$_{14}$-14-2 (uncloned) viruses

15   were used for comparison. Both chimeric viruses reached a maximal virus
yield of approximately one log higher than either parental virus. In the case of
the YF/JE SA$_{14}$-14-2 chimera, the peak of virus production occurred 12 hours
later than the YF/JE Nakayama chimera (50 hours vs. 38 hours). The YF/JE
Nakayama chimera exhibited considerably more cytopathic effects than the

20   YF/JE SA$_{14}$-14-2 chimera on this cell line. A similar experiment was carried
out in C6/36 cells after low multiplicity infection (0.5 plaque-forming
units/cell). Fig. 2 also illustrates the growth kinetics of the viruses in this
invertebrate cell line. Similar virus yields were obtained at all points used for
virus harvest in this experiment, further substantiating the notion that chimeric

25   viruses are not impaired in replication efficiency.

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week post-inoculation. No mortality or illness was observed among mice

receiving either the JE SA$_{14}$-14-2 parent or the chimera. The inocula used for

the experiments were titered at the time of injection and a subgroup of the

surviving mice were tested for the presence of neutralizing antibodies to

5   confirm that infection had taken place. Among those tested, titers against the

JE SA$_{14}$-14-2 virus were similar for animals receiving either this strain or the

chimera.

          The results of additional experiments investigating the

neurovirulence of the YF/JE SA$_{14}$-14-2 chimera in mice are illustrated in Table

10   4. In these experiments, all of the mice inoculated with YF5.2iv died within 7-

8 days. In contrast, none of the mice inoculated with YF/JE SA$_{14}$-14-2 died

during two weeks of post-inoculation observation.

          The results of experiments investigating the neuroinvasiveness and

pathogenesis of YF/JE chimeras are illustrated in Table 5. In these

15   experiments, the chimeric viruses were inoculated into 3 week old mice at

doses varying between 10,000 and 1 million plaque-forming units via the

intraperitoneal route. None of the mice inoculated with YF/JE Nakayama or

YF/JE SA$_{14}$-14-2 died during three weeks of post-inoculation observation,

indicating that the virus was incapable of causing illness after peripheral

20   inoculation. Mice inoculated with YF/JE SA$_{14}$-14-2 developed neutralizing

antibodies against JE virus (Fig. 7).

Construction of cDNA Templates for Generation of Yellow Fever/Dengue

(YF/DEN) Chimeric Viruses

          Derivation of chimeric Yellow Fever/Dengue (YF/DEN) viruses is

25   described as follows, which, in principle, is carried out the same as construction

of the YF/JE chimera described above. Other flavivirus chimeras can be

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MON310 furnished by Dr. Wright. PCR primers included a 5' primer flanking

the *Sph*I site and a 3' primer homologous to the DEN-2 nucleotides

immediately upstream of the signalase site at the E/NSI junction and replacing

the signalase site by substitutions that create a novel site, but also introduce a

5   *Nar*I site. The resulting 1,170 basepair PCR fragment was then introduced into

YFM5.2(*Nar*I[+]*Sph*I[-]).

        The 5' portion of the DEN-2 clone including the prM and amino

terminal portion of the E protein was engineered into the YF5'3'IV plasmid

using a chimeric PCR primer. The chimeric primer, incorporating the 3' end of

10   negative-sense YF C protein and 5' end of DEN-2 prM protein, was used with a

positive-sense primer flanking the SP6 promoter of the YF5'3'IV plasmid to

generate a 771 basepair PCR product with a 20 base extension representing

DEN-2 prM sequence. This PCR product was then used to prime the DEN-2

plasmid in conjunction with a 3' primer representing DEN-2 sequence 1,501-

15   1,522 and flanking the *Sph*I, to generate an 1,800 basepair final PCR product

including the YF sequence from the *Not*I site through the SP6 promoter, YF 5'

untranslated region, and YF C protein, contiguous with the DEN-2 prM-E1522

sequence. The PCR product was ligated into YF5'3'IV using *Not*I and *Sph*I

sites to yield the YF5'3'IV/DEN(prM-E) plasmid.

20   *Construction of Chimeric Templates for Other Flaviviruses*

        Procedures for generating full-length cDNA templates encoding

chimeric YF/MVE, YF/SLE, YF/WN, YF/TBE viruses are similar to those

described above for the YF/DEN-2 system. Table 6 illustrates the features of

the strategy for generating YF17D-based chimeric viruses. The unique

25   restriction sites used for *in vitro* ligation, and the chimeric primers for

engineering the C/prM and E/NSI junctions are also shown. Sources of cDNA

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this protein may abrogate the immunity to YF associated with antibodies

against NS1, and thus avoid problems with vector immunity if more than one

chimeric vaccine was to be needed in a given recipient, or if a YF vaccine had

been previously given or needed at a future point.

5   The strategy involves creating a series of in-frame deletions within

the NS1 coding region of the YFM5.2 plasmid, in conjunction with engineering

a translational termination codon at the end of E, and a series of two IRESs

(internal ribosome entry sites). One IRES is immediately downstream of the

termination codon and allows for expression of an open reading frame within

10   the region between E and NS1. The second IRES initiates translation from

truncated NS1 proteins, providing expression of the remainder of the YF

nonstructural polyprotein. These derivatives are tested for recovery of

infectious virus and the construct with the largest deletion is used for insertion

of foreign sequences (*e.g.*, HCV proteins) in the first IRES. This particular

15   construct can also serve as a basis for determining whether deletion of NS1 will

affect vector-specific immunity in the context of YF/Flavivirus chimeric

viruses expressing prM-E, as described above.

           The insertion of nucleotides encoding E1, E2, and/or E1 plus E2

HCV proteins is limited by the size of the deletion tolerated in the NS1 protein.

20   Because of this, truncated HCV proteins can be used to enhance stability within

the modified YF clone. The HCV proteins are engineered with an N-terminal

signal sequence immediately following the IRES and a termination codon at the

C terminus. This construction will direct the HCV proteins into the

endoplasmic reticulum for secretion from the cell. The strategy for this

25   construction is shown schematically in Fig. 6. Plasmids encoding HCV

proteins of genotype I can be used for these constructions, for example, HCV

plasmids obtained from Dr. Charles Rice at Washington University (Grakoui *et*

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Table 1

Characterization of YF/JE chimeras

	Clone	Yield (µg)	Infectivity plaques/100ng LLC-MK2	PBS log titer VERO	RNAse log titer VERO	DNAse log titer VERO
	YF5.21v	5.5	15	7.2	0	7
5	YF/JE-S	7.6	50	6.2	0	6.2
	YF/JE-N	7	60	5	0	5.4

Table 2

Sequence comparison of JE strains and YF/JE chimeras

	Virus	E 107	E 138	E 176	E 177	E 227	E 243	E 244	E 279
10	JE SA14-14-2	F	K	V	T	S	K	G	M
	YF/JE SA14-14-2	F	K	V	A	S	E	G	M
15	YF/JE NAK	L	E	I	T	P	E	E	K
	JE NAK	L	E	I	T	P	E	E	K
	JE SA14	L	E	I	T	S	E	G	K

Table 3

Plaque reduction neutralization titers on YF/JE chimeras

	Virus	non-immune ascitic fluid	YF ascitic fluid	JE ascitic fluid	non-immune IgG	YF IgG
20	YF5.2iv	<1.3	3.7	<1.3	<2.2	>4.3
	JE SA14-14-2	<1.3	<1.3	3.4	<2.2	<2.2
	YF/JE SA14-14-2	<1.3	<1.3	3.1	<2.2	<1.9
25	YF/JE Nakayama	<1.3	<1.3	3.4	<2.2	<2.2

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Table 6

Engineering of YF/Flavivirus chimeras

Virus	Chimeric C/prM junction[1]	Chimeric E/NS1 junction[2]	5' ligation[3]	3' ligation[4]	Sites[5] eliminated or (created)
YF/WN	X-cactgggagagcttgaaggtc (SEQ ID NO:1)	aaagccagttgcagccgcggtttaa (SEQ ID NO:2)	AatII	NsiI	
YF/DEN-1	X-aaggtagactggtgggctccc (SEQ ID NO:3)	gatcctcagtaccaaccgcggtttaa (SEQ ID NO:4)	AatII	SphI	SphI in DEN
YF/DEN-2	X-aaggtagattggtgtgcattg (SEQ ID NO:5)	aaccctcagtaccaccgcggtttaa (SEQ ID NO:6)	AatII	SphI	
YF/DEN-3	X-aaggtgaattgaagtgctcta (SEQ ID NO:7)	accccagcaccaccgcggtttaa (SEQ ID NO:8)	AatII	SphI	XhoI in DEN (SphI in DEN)
YF/DEN-4	X-aaaaggaacagttgttctcta (SEQ ID NO:9)	acccgaagtgtcaaccgcggtttaa (SEQ ID NO:10)	AatII	NsiI	
YF/SLE	X-aacgtgaatagttggatagtc (SEQ ID NO:11)	accgttggtcgcacccgcggtttaa (SEQ ID NO:12)	AatII	SphI	AatII in SLE
YF/MVE	X-aatttcgaaaggtggaaggtc (SEQ ID NO:13)	gaccggtgtttacagccgcggtttaa (SEQ ID NO:14)	AatII	AgeI	(AgeI in YF)
YF/TBE	X-tactgcgaacgacgttgccac (SEQ ID NO:15)	actgggaacctcacccgcggtttaa (SEQ ID NO:16)	AatII	AgeI	(AgeI in YF)

1,2: The column illustrates the oligonucleotide used to generate chimeric YF/Flavivirus primers corresponding to the C/prM or E/NS1 junction. (See text). X = carboxyl terminal coding sequence of the YF capsid. The underlined region corresponds to the targeted heterologous sequence immediately upstream of the NarI site (antisense - ccgcgg). This site allows insertion of PCR products into the Yfm5.2 (NarI) plasmid required for generating full-length cDNA templates. Other nucleotides are specific to the heterologous virus. Oligonucleotide primers are listed 5' to 3'.

3,4: The unique restriction sites used for creating restriction fragments that can be isolated and ligated in vitro to produce full-length chimeric cDNA templates are listed. Because some sequences do not contain convenient sites, engineering of appropriate sites is required in some cases (footnote 5).

5: In parentheses are the restriction enzyme sites that must be created either in the YF backbone or the heterologous virus to allow efficient in vitro ligation. Sites not in parentheses must be eliminated. All such modifications are done by silent mutagenesis of the cDNA for the respective clone. Blank spaces indicate that no modification of the cDNA clones is required.

Other Embodiments

Other embodiments are within the following claims. For example, the prM-E protein genes of other flaviviruses of medical importance can be

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response. The vaccine can be administered as a primary prophylactic agent in adults or children at risk of flavivirus infection. The vaccines can also be used as secondary agents for treating flavivirus-infected patients by stimulating an immune response against the flavivirus.

5   It may be desirable to use the yellow fever vaccine vector system for immunizing a host against one virus (for example, Japanese Encephalitis virus) and to later reimmunize the same individual against a second or third virus using a different chimeric construct. A significant advantage of the chimeric yellow fever system is that the vector will not elicit strong immunity to itself.

10   Nor will prior immunity to yellow fever virus preclude the use of the chimeric vaccine as a vector for heterologous gene expression. These advantages are due to the removal of the portion of the yellow fever vaccine E gene that encodes neutralizing (protective) antigens to yellow fever, and replacement with another, heterologous gene that does not provide cross-protection against

15   yellow fever. Although YF17D virus nonstructural proteins may play a role in protection, for example, by eliciting antibodies against NS1, which is involved in complement-dependent antibody mediated lysis of infected cells (Schlesinger et al., J. Immunology 135:2805-2809, 1985), or by inducing cytotoxic T cell responses to NS3 or other proteins of the virus, it is unlikely

20   that these responses will abrogate the ability of a live virus vaccine to stimulate neutralizing antibodies. This is supported by the facts that (1) individuals who have been previously infected with JE virus respond to vaccination with YF17D similarly to persons without previous JE infection, and (2) individuals who have previously received the YF17D vaccine respond to revaccination

25   with a rise in neutralizing antibody titers (Sweet et al., Am. J. Trop. Med. Hyg. 11:562-569, 1962). Thus, the chimeric vector can be used in populations that are immune to yellow fever because of prior natural infection or vaccination,

-29-

(Chambers *et al.* (1990) "Flavivirus Genome Organization, Expression, and Replication," In *Annual Review of Microbiology* 44:649-688), providing an important safety measure.

All references cited herein are incorporated by reference in their

5   entirety.

What is claimed is:

-31-

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACTGGGAGA GCTTGAAGGT C   21

      (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 25 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAAGCCAGTT GCAGCCGCGG TTTAA   25

      (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAGGTAGACT GGTGGGCTCC C   21

      (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 26 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

-33-

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCCCCAGCA CCACCCGCGG TTTAA   25

     (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAAAGGAACA GTTGTTCTCT A   21

     (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 25 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACCCGAAGTG TCAACCGCGG TTTAA   25

     (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AACGTGAATA GTTGGATAGT C   21

     (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 25 base pairs

-35-

    (2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 25 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACTGGGAACC TCACCCGCGG TTTAA   25

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mutation that prevents prM cleavage to produce M protein.

    7. The chimeric virus of claim 1, wherein the signal sequences at the
C/prM and E/NS1 junctions are maintained in construction of said chimeric
flavivirus.

5   8. Use of a chimeric live, infectious, attenuated virus in the
preparation of a medicament for preventing or treating flavivirus infection in a
patient, wherein the chimeric, live, infectious attenuated virus comprises
        a yellow fever virus in which the nucleotide sequence encoding a
prM-E protein is either deleted, truncated, or mutated so that functional yellow
10   fever virus prM-E protein is not expressed, and
        integrated into the genome of said yellow fever virus, a nucleotide
sequence encoding a prM-E protein of a second, different flavivirus, so that
said prM-E protein of said second flavivirus is expressed.

    9. The use of claim 8, wherein said second flavivirus is a Japanese
15   Encephalitis (JE) virus.

    10. The use of claim 8, wherein said second flavivirus is a Dengue
virus selected from the group consisting of Dengue types 1-4.

    11. The use of claim 8, wherein said second flavivirus is selected
from the group consisting of a Murray Valley Encephalitis virus, a St. Louis
20   Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus, a
Hepatitis C virus, a Kunjin virus, a Central European Encephalitis virus, a
Russian Spring-Summer Encephalitis virus, a Powassan virus, a Kyasanur

1-4.

18. The nucleic molecule of claim 15, wherein said second flavivirus is selected from the group consisting of a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne

5   Encephalitis virus, a Hepatitis C virus, a Kunjin virus, a Central European Encephalitis virus, a Russian Spring-Summer Encephalitis virus, a Powassan virus, a Kyasanur Forest Disease virus, and an Omsk Hemorrhagic Fever virus.

19. The nucleic acid molecule of claim 15, wherein the nucleotide sequence encoding the prM-E protein of said second, different flavivirus

10   replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus.

20. The nucleic acid molecule of claim 15, wherein said nucleotide sequence encoding said prM-E protein of said second, different flavivirus comprises a mutation that prevents prM cleavage to produce M protein.

15   21. The nucleic acid molecule of claim 15, wherein the signal sequences at the C/prM and E/NS1 junctions are maintained in construction of said chimeric flavivirus.
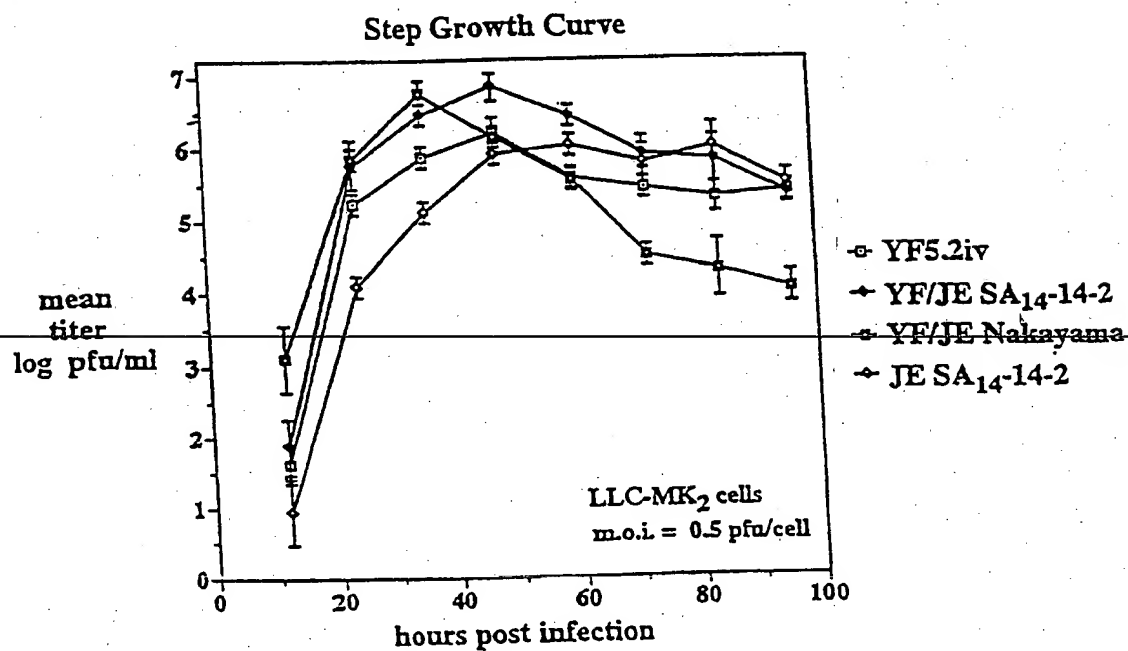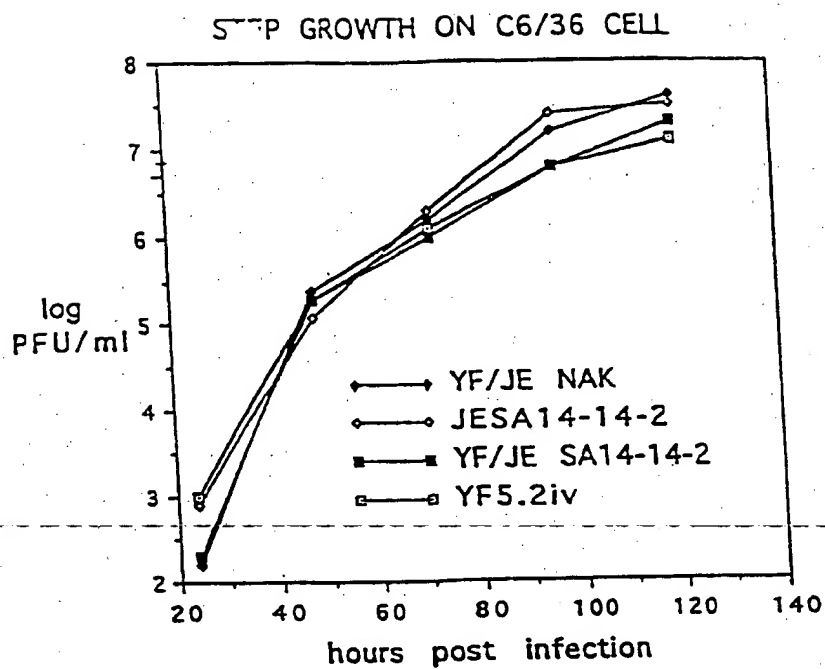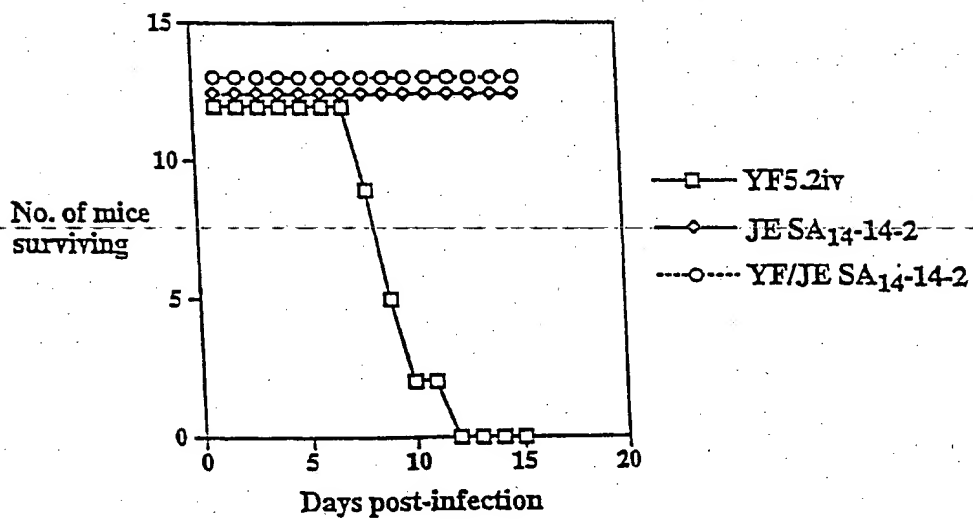
STEP GROWTH ON C6/36 CELL

STEP GROWTH ON C6/36 CELL

log PFU/ml

→ YF/JE NAK
→ JESA14-14-2
→ YF/JE SA14-14-2
→ YF5.2iv

hours post infection

Step Growth Curve

mean titer log pfu/ml

LLC-MK₂ cells
m.o.i = 0.5 pfu/cell

hours post infection

-□- YF5.2iv
-◆- YF/JE SA$_{14}$-14-2
-○- YF/JE Nakayama
-◇- JE SA$_{14}$-14-2

FIG. 2

Mouse neurovirulence analysis

MICE: 4 week old ICR males/females
VIRUS DOSE: 10^4 pfu intracerebrally

Virus	Survival	P
YF5.2iv	0/12 (0%)	-
JE SA$_{14}$-14-2	12/12 (100%)	<0.001
YF/JE SA$_{14}$-14-2	13/13 (100%)	<0.001

Fig. 4

Structure of modified YF clones expressing
E/NS1 intergenic open reading frames
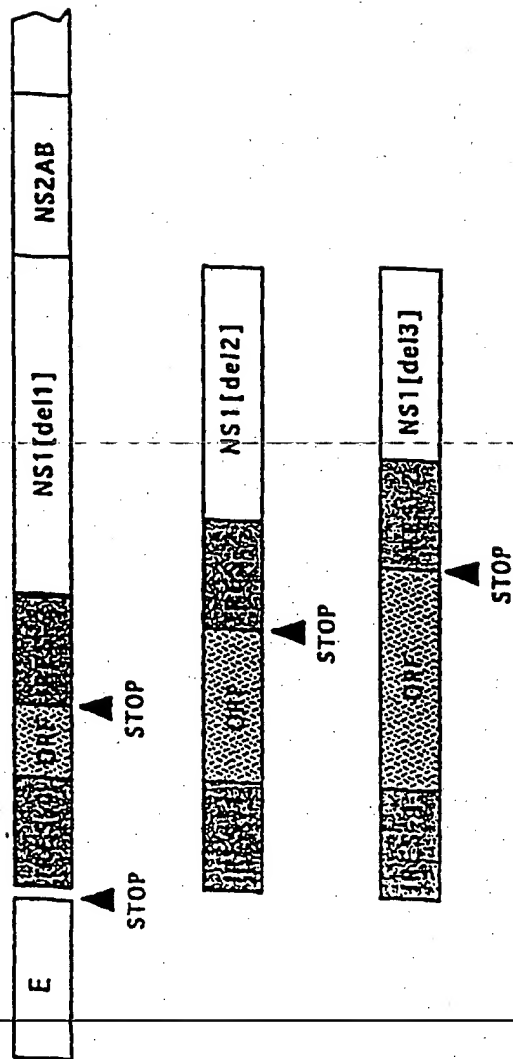
NS2AB

NS1[del1]

STOP

E

STOP

NS1[del2]

STOP

NS1[del3]

STOP

Fig. 6

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/12, 39/193; C12N 7/01
US CL   :424/199.1, 218.1; 435/235.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   424/199.1, 218.1; 435/235.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE
search terms:Japanese encephalitis, attenuat?, yellow fever, chimer?, dengue, flavivir?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BRAY et al. Construction of Intertypic Chimeric dengue Viruses by Substitution of Structural Protein Genes. November 1991. Proc. Natl. Acad. Sci., USA. Vol. 88, pages 10342-10346, see entire document.	1-21
Y	VENUGOPAL et al. Towards a New Generation of Flavivirus Vaccines. Vaccine. 1994. Vol. 12, No. 11, pages 966-975, see entire document.	1-21
Y	MARCHEVSKY et al. Phenotypic Analysis of Yellow Fever Virus Derived From Complementary DNA. American J Tropical Medicine & Hygiene. 1995. Vol. 52, No. 1, pages 75-80, see entire document..	1-21

☐ Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier document published on or after the international filing date	
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 APRIL 1998	23 JUN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MP WOODWARD
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